MICROSCOPY AND ANALYSIS

Life Science Microscopy Optics

Essential Knowledge Briefings

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Fluorescence microscopy, as well as many other imaging methods, have very specific optical requirements. Dedicated objectives are key for getting versatile, detail-rich images, such as the one from the Rainbow Mouse. Adapted with permission from Macmillan Publishers Ltd·Tanaka T. Komai Y, Tokuyama Y, ***et al. Nature Cell Biology*** 2013;15:511–9, copyright 2013.

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About Essential Knowledge Briefings

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INTRODUCTION

Perhaps more than any other scientific instrument, the microscope has opened our eyes to the sheer amount of beauty and complexity in the world. Without it, we would never have known about the existence of biological cells, minerals, molecules and microbes – indeed, just about anything smaller than the width of a human hair. Ever since optical microscopes first became widely used in the 17th century, progress in the sciences, and the life sciences in particular, has been rapid – and a large portion of this can in some way be attributed to microscopy.

The first microscopes were simple, if transformative, devices. But as time has gone on, microscopy has become more refined, more complex and more specialist. Today, just within the field of optical microscopy, there are dozens of specialist techniques, all with their own particular characteristics and components. Each of these techniques has a single goal, however: viewing tiny things clearly. Nonetheless, when beginning to use microscopy seriously for the first time, it can be daunting to know where to start.

This Essential Knowledge Briefing (EKB) aims to give newcomers an introduction to modern optical microscopy. It will detail the origins and history of optical microscopes and explain how they work. It will also go over the most widely used specialist techniques in life science microscopy, with an emphasis on the important role played by objective lenses: the one interchangeable component that can most improve image quality.

HISTORY AND BACKGROUND

If you were an educated person living in late 17th century England, you might well have owned a copy of ***Micrographia***. Not only was this 1665 tome the first 'coffee table' science book, it was also the first book to popularize the then new science of microscopy. Its author, the English polymath Robert Hooke, had used an early, primitive microscope to produce the first detailed drawings of minuscule organisms, notably insects such as fleas and lice. Among several of the revelations inside the book was Hooke's coining of the biological term 'cell' as the smallest functional unit of an organism.

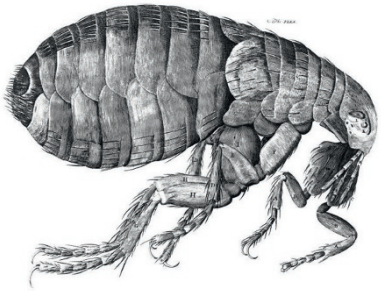

Figure 1. Hooke's drawing of a flea

One of the early readers of ***Micrographia*** was the Dutch scientist and tradesman Anton van Leeuwenhoek, who was sufficiently inspired to begin grinding his own lenses and creating his own microscopes. Simple yet powerful, these microscopes allowed van Leeuwenhoek to go one step further than Hooke and observe single-celled organisms, which he called 'animalcules' (and which today we call micro-organisms). Though his contemporaries were initially skeptical, van Leeuwenhoek's observations were eventually accepted, and he came to be known as the Father of Microbiology.

As microscopes became more widely available, people were continually astounded by their new window onto the microscopic world. But it was not always such a clear window, and imperfections in the optics often made resolving a specimen's tiniest details frustratingly difficult. Indeed, for some 200 years, manufacturing a lens was largely a process of trial and error.

Things began to change in the 1840s. Carl Zeiss, a German instrument manufacturer, set up a workshop to radically improve the performance of microscopes. As he refined his designs, his reputation grew, allowing him to sell more than 1000 units over a period of 20 years. He collaborated with a glass chemist, Otto Schott, to produce a new type of glass that could eradicate many of the optical aberrations that had bedeviled earlier types of lens. He also teamed up with a physicist, Ernst Abbe, who was to lay down a mathematical basis for optics that is still in use today.

One of Abbe's mathematical formulae is so important that it is engraved on a memorial in Jena, the city in Germany where he worked at the Zeiss firm. This formula describes the maximum amount of detail that any optical system, such as a microscope, can resolve (see page 11). One might imagine Abbe to have been disappointed to discover this resolution limit, but it actually allowed him and his colleagues to design even better lenses – ones that can magnify as much as is theoretically possible.

But sometimes even features above Abbe's resolution limit could not be seen. Imagine, for example, observing through a microscope a specimen that is naturally transparent. It is impossible to make out the features, even if they have been resolved by the lens, because they are transmitting light of similar intensity to their surroundings – simply put, they are camouflaged. This is

a problem of contrast, and it is one that microscope developers began to address in the early 20th century and have continued to address to this day, with all sorts of specialist techniques. The main techniques to improve contrast in microscopy are described in the *Specialist microscopy techniques* chapter.

Microscope optics in practice

The simplest type of microscope is a magnifying glass. A single lens bends or 'refracts' the light coming off an object so that it covers a larger area of the retina at the back of your eye than would normally be the case. This enlarged image appears to exist on the far side of the lens, like the object itself, even though you can't see it if you look round the side. For this reason it is called a ***virtual image*** (Figure 2A).

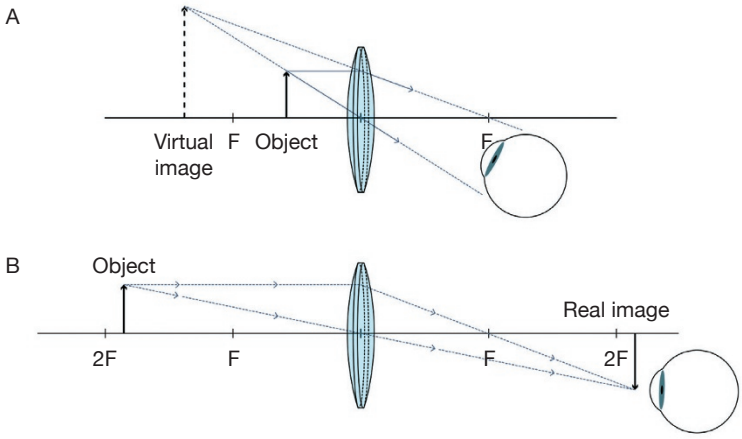

Figure 2. A represents a virtual image, while B depicts a real image. Copyright Olympus.

Children, of course, often have a less scientific use for magnifying glasses: concentrating the sun's rays to burn

things. The resultant image (the smoldering dot) is the actual point where the light converges, therefore it is called a ***real image*** (Figure 2B). In this instance, the location of the image is also the ***focal point*** of the lens, defined as the point onto which the lens focuses parallel rays of light, while the distance from the center of the lens to the focal point is known as the ***focal length***. A point source of light needs to be infinitely far away for its rays to be truly parallel when they arrive at the lens, but rays from the sun provide a close enough approximation. The focal length is a fundamental property of a lens – in microscopy, the shorter a lens's focal length, the larger its magnification.

Most magnifying lenses can magnify an object two or three times its original size, although the very best ones can reach 30x magnification. To magnify objects further, scientists use a ***compound microscope*** and this is the type you see most often in the laboratory. The simplest compound microscope has two lenses: an ***objective lens*** (Figure 3A), which sits next to the object being imaged, and an ***eyepiece lens*** or 'ocular', which you look through and which further enlarges the images created by the objective lens.

The objective lens, held a small ***working distance*** from the cover-slip that houses the specimen being studied, forms an image of the specimen that is on the same side as the eyepiece lens. Crucially, this is a real image, which means that it can be magnified again by the eyepiece before it reaches your eye, producing a virtual image.

But this is only the simplest arrangement. Since the 1980s, almost all serious microscope manufacturers have employed an intermediate ***tube lens*** between the objective and the eyepiece

lenses. The light rays between the objective and tube lenses are parallel, which means they are focused at infinity; in other words, they are ***infinity corrected*** (Figure 3B).

Infinity correction allows a greater distance between the lenses, so that specialist optical accessories, such as polarizers or prisms, can be easily inserted without distorting the image quality. Projecting the images into infinity also provides a more comfortable viewing experience, because the human eye is more relaxed when looking into the distance. This allows users to spend several hours in front of a microscope without suffering from eye strain.

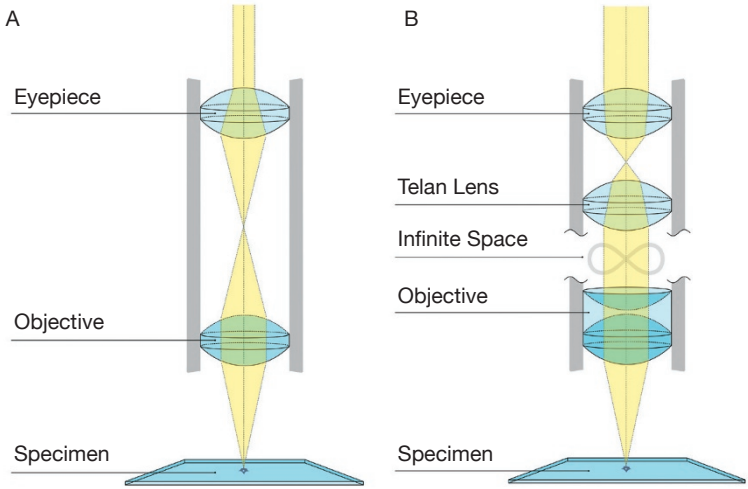

Figure 3. How a compound microscope works. A shows a former finite corrected microscope. These microscopes had a defined tubelength, meaning that the distance between the objective lens and eyepiece was fixed. Every insertion between the objective and the eyepiece would have distorted the image due to the different diffractive properties of the different wavelengths of the visible light spectrum. B shows an infinite corrected microscope. These have a parallel light path between the objective and Telan lens, known as the infinite space, which allows optical parts to be inserted without distorting the image. This enables a variety of different observation methods which require different optical parts in the beampath of the infinite space. Copyright Olympus.

Figure 4. Example of a compound microscope. Copyright Olympus..

Figure 4 shows a compound microscope. The total magnification of a compound microscope is much greater than a magnifying glass can deliver because it is a product of the magnifications of both the objective lens and the eyepiece lens. For example, an objective lens that has 20x magnification coupled with an eyepiece lens that has 10x magnification produces a total magnification of 200x.

But greater magnification isn't always a priority. Generally, the greater the magnification, the smaller the portion of the object being seen; that is, the smaller the ***field of view***. Furthermore, a greater magnification does not always reveal more details of the specimen. As discovered by Abbe, the resolving power of a light microscope, meaning how close two features on a specimen can be to each other and still be perceived as separate entities, is solely dependent on the ***numerical aperture*** (NA) of the objective lens and the wavelength of light illuminating the specimen. The NA is simply a measure of the amount of light that a lens can

gather from its surroundings. Abbe came up with a now-famous equation that describes the relationship:

$$r = \frac{0.61\lambda}{NA}$$

Where r is the resolution, λ is the wavelength and NA is the numerical aperture.

The NA doesn't just depend on the shape of the lens, but also on the medium that separates it from the specimen. This medium is often simply air, but some objectives are designed to work with an ***immersion medium***, such as oil, water or silicone, which is applied between the specimen and the lens. The immersion medium is designed to have a light-bending power, or refractive index, that is similar to that of the specimen being studied, which depends on how the specimen is prepared and presented. This ensures that light leaving the specimen travels to the objective as if there has been no change in medium at all. As a result, no light is bent, or refracted, away from the objective, increasing the NA of the objective and thus the resolving power of the microscope.

Illumination is crucial in creating high-quality images. It is normally provided by a lamp that is housed behind the microscope, either at the bottom or the top. The light from the lamp is guided towards the stage through mirrors and lens systems, the last of which is known as the ***condenser***. Just as the image of the object must be brought into focus, so too must the lamp light be focused; the accepted method of doing this is known as ***Köhler illumination***. If the illuminating light is set up according to this method, the object will be illuminated evenly, without glare or other artifacts.

Objectives

More so than any other component of a microscope, the objective lens determines image quality, and that is fortunate, because, apart from the eyepieces, it is the one magnifying component that can be readily changed. Cheap objectives cause far more optical aberrations than expensive ones, distorting the observed image. High-quality objectives can cost hundreds if not thousands of dollars and are highly complex components, often hand-crafted with great expertise.

There are two main types of optical aberration. The first, known as ***chromatic aberration***, occurs because the spectrum of colors that makes up white light is refracted in a lens to varying degrees. The effects of this aberration can be seen around any edges in an image, which appear 'fringed' with different colors. The second type of aberration, known as ***spherical aberration***, occurs because of imperfections in the shape of a lens. For instance, in a convex lens – the bulging sort – the light entering around the edges can come to a focus after a shorter distance than the light entering the middle. The result is an image that appears slightly blurry, even if it is properly focused.

To avoid such aberrations in a final image, a single objective 'lens' must in fact house several lens elements, which correct for one another's deficiencies. Generally speaking, the more expensive the objective, the more lens elements it will house, the more refined those lens elements, and the greater the level of aberration correction.

Typically, three levels of aberration correction are available in microscopy, using objectives that differ in the number of lens elements they contain (Figure 5).

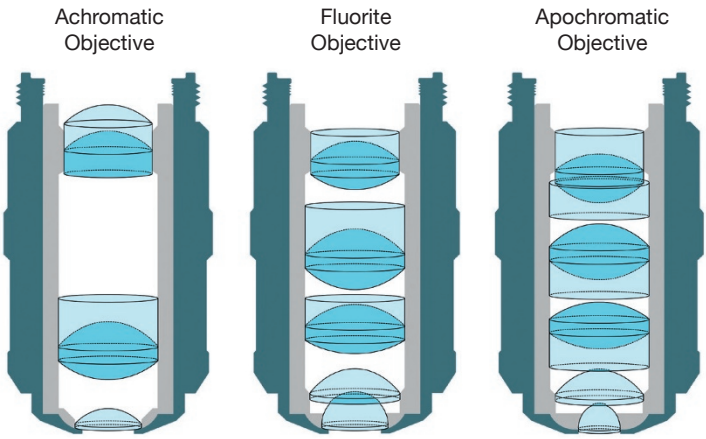

Figure 5. Optical correction in objectives. Copyright Olympus.

- **Plan-achromatic objectives: the lowest level of correction**
  These lenses provide field flatness with dark-field and bright-field observations over the complete field of view in transmitted light. Chromatically, they correct for two colors (blue and red). They are generally suited for routine laboratory and examination work, as well as for image acquisition.
- **Fluorites or semi-apochromatic objectives: a medium level of correction**
  Lenses of this level of correction display flat images with high transmissions throughout the visible spectrum of light. Chromatically, they correct for three colors (violet, blue and red), although there is still very slight aberration in the far violet area. They provide good image quality in fluorescence, bright-field and differential interference contrast (DIC; also known as Nomarski microscopy) observations.

- **Apochromatic objectives: the highest level of correction** These high-end lenses provide full compensation for both spherical and chromatic aberrations from the ultraviolet to the infrared region of the light spectrum. They produce images of the highest quality, particularly during observations deep inside living tissue.

The level of aberration correction, as well as other lens properties such as magnification, working distance and NA are inscribed on the lens barrel (Figure 6).

Figure 6. Lens barrel showing the inscriptions detailing the lens properties. Copyright Olympus.

SPECIALIST MICROSCOPY TECHNIQUES

Bright-field microscopy

The classic way of using a microscope involves illuminating a specimen from directly opposite the objective. Some of the physical properties of this illuminating light, including its color and intensity, are altered by passage through the specimen, while light that doesn't pass through the specimen remains unaltered. This produces an image of the specimen on a bright background, hence the name of the technique – ***bright-field microscopy***.

Objectives for bright-field microscopy need to feature good spherical correction over the complete field of view, with minimal chromatic aberration effects at the edges of the field of view.

Dark-field microscopy

The trouble with bright-field microscopy is that it is inherently low-contrast, particularly when applied to specimens that are transparent or colorless. With such specimens, it is possible to boost contrast by staining key components with colored dyes prior to observation. If this is not possible or convenient, however, there is a simple alternative, which involves blocking out the axial light and illuminating from the side (obliquely). This technique results in an image of a bright specimen on a dark background, hence its name – ***dark-field microscopy***.

There are two ways to block out the axial light, both of which can be applied to a regular bright-field microscope. One is to insert an opaque circular filter beneath the condenser; another is to employ a condenser designed specifically for dark-field imaging. Either way, the axial light from the illuminating source is eclipsed, allowing only oblique rays to pass through to the specimen stage. If no specimen was present, these rays would career past the objective, leaving the

image completely dark. Only with the specimen in place are some of the rays scattered towards the objective.

Scientists turn to dark-field microscopy to observe particular aspects of a specimen (internal edges, for example), that appear strongly only when the direct glare of the illuminating light is removed. Generating a reasonable image with dark-field microscopy requires very strong initial illumination, however, which risks harming delicate specimens.

Objectives for dark-field microscopy require similar properties to those used for bright-field microscopy. In addition, the relation between the NA of the condenser and that of the objective is very important. If the NA of the objective is higher than that of the condenser, illumination light is able to enter the objective and swamp the scattered light from the specimen, making dark-field microscopy impossible.

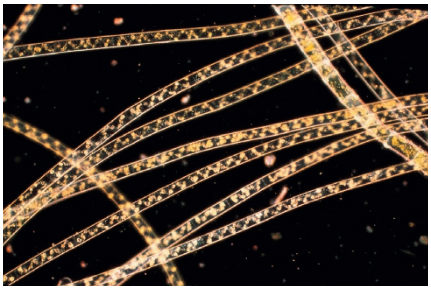

Figure 7. Algae imaged by dark-field microscopy. Copyright Olympus.

Phase-contrast microscopy

Fortunately, dark-field microscopy is not the only way to boost contrast, as the Nobel prize-winning physicist Frits Zernike discovered in the early 1930s. Rather than simply block the direct light, Zernike took advantage of the fact that light slows down

slightly as it travels through a transparent object, causing it to become 'phase-shifted' compared to an identical wave of light that didn't pass through the object.

A useful analogy is to picture water waves from the side. Take two sets of identical waves, with one set travelling slightly ahead of the other, and then combine them. Depending on how the peaks and troughs line up, the two sets may 'destructively interfere' with each other, where the peaks of one set combine with the troughs of the other to produce calm, flat water. Alternatively, the peaks in one set may line up with the peaks of the other. In this case there is 'constructive interference' – extra-big waves or extra-deep troughs.

By modifying the phase-shift between the direct and scattered light from the specimen, ***phase-contrast microscopy*** is able to produce high-contrast images, darkening the shadows and brightening the highlights. To perform phase-contrast microscopy, specialist phase-shifting condensers and objectives must be fitted to a microscope. These objectives have an integrated phase plate and phase contrast only works if the matching phase annulus is inserted in the illumination condenser.

Polarized-light microscopy

Another way to improve contrast is to exploit the polarization of light. A normal, unpolarized light wave can oscillate in all directions perpendicular to its direction of travel. Passing this light wave through a polarizing filter confines these oscillations to just a single direction, causing it to become plane-polarized.

In ***polarized-light microscopy***, one polarizing filter is inserted beneath the condenser and another above the objective. This can be done on a regular microscope or on a dedicated polarized-light

microscope. Normally if the planes of the two polarizing filters are in a 90° relative orientation, the so-called crossed polarizer orientation, you would see no light through the eyepiece at all because the first filter would confine the light wave to oscillating in one direction, which would then be blocked by the second orthogonal filter. However, some light can make it through to the eyepiece if the specimen between the polarizers exhibits some degree of 'birefringence'.

Birefringent specimens are crystalline materials such rocks, bones and hair with asymmetric crystal structures, causing the single-incident light wave to split in two. The two resultant waves are plane-polarized at right angles to each other, with their precise orientation determined by the specimen's crystal structure. As a result of this splitting and re-orientation, the light leaving the birefringent specimen can now pass through the second polarizer, because that light's plane of polarization is no longer exactly orthogonal to the two new plane-polarized rays.

More importantly, those two rays, having taken slightly different routes through the specimen, become slightly out of phase with each other. As in phase-contrast microscopy, the interference that results from this phase-shift generates a high-contrast image.

Figure 8. Crystalline structures imaged by polarized-light microscopy. Copyright Olympus.

Because it is essential that the objective does not alter the polarization of the light at all, polarization light microscopy requires special strain-free lenses.

Nomarski differential interference contrast microscopy

Polarized-light microscopy has been in use since the late 1930s, but since then more specialist versions of it have been developed. One of these is *DIC* microscopy, which was invented in the mid-1950s by the French optician Georges Nomarski. The technique still requires plane-polarized light to be split in two, but not by birefringent specimens. Instead, this is achieved by placing a special 'Wollaston' prism just above the lower polarizing filter in a regular microscope. The Wollaston prism splits the plane-polarized light into two new plane-polarized rays that travel parallel and very close to each other through the specimen. Between the objective and the second polarizing filter, another Wollaston prism recombines them.

In DIC microscopy, unlike in regular polarized-light microscopy, the specimen itself does not split the illuminating light. Nonetheless, the twin rays still become phase-shifted relative to each other as they travel through slightly different parts of the specimen – parts that have steep gradients of refractive index, *eg* cell boundaries.

Once the rays are recombined and processed through the second polarizer, the phase-shift again generates interference, although of a rather strange sort. Along any edge within the specimen's image, one side typically appears darker than, or a different color to, the adjacent side. The result is that the overall image appears to be raised above the background, giving the appearance of depth, or relief.

This relief is not real, but an artifact of the imaging process, therefore it cannot be used to estimate actual heights or depths. Still, there are various benefits to DIC microscopy, in addition to being able to work with samples that aren't birefringent. Unlike phase-contrast microscopy, DIC microscopy can employ the full NA of the objective, allowing very high resolution. It is also free of imaging artifacts known as halos – bright rings that can appear around the peripheries of objects in phase-contrast microscopy.

A limitation of DIC microscopy, however, is that it works best when specimens are housed in glass dishes, because plastic dishes degrade the image quality.

Hoffman modulation contrast microscopy

Hoffman modulation contrast microscopy, invented by Robert Hoffman and Leo Gross of the Waldemar Medical Research Foundation in New York, USA, in 1975, also generates images with pseudo-relief, but in a different way to DIC microscopy. It relies on two specialist components – a 'slit plate' beneath the condenser and a 'modulator plate' above the objective.

The slit plate, which is blacked-out except for a small slit located off-center, allows the illuminating light to pass through a specimen only at an oblique angle. Depending on what happens to

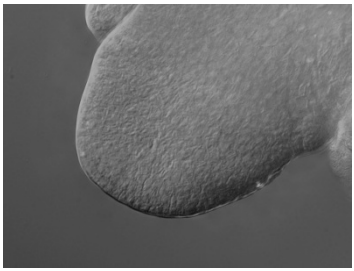

Figure 9. A shoot apical meristem of rice. Imaged by Hoffman modulation. Copyright Olympus.

it as it passes through the specimen, the light then strikes different parts of the modulator plate, which is tinted with black, gray and clear bands. If the light passes straight through the specimen, it strikes the modulator's gray band. Meanwhile, any light that is deflected by the specimen strikes one of the other two bands – light deflected one way strikes the black band, while light deflected the other way strikes the clear band.

By tinting (or not) the deflected light in this way, the parts of the specimen that cause the deflection – for example, parts that are thinner or thicker than usual – appear in the image darker or lighter than the neighboring parts. This produces a high-contrast image superimposed with a relief effect. The image can be improved by situating a pair of polarizing filters beneath the condenser: as they are brought towards a crossed (90°) relative orientation, the aperture of the slit plate effectively narrows. This modification results in even greater contrast, although it is not best suited for thicker specimens.

One particular advantage of Hoffmann modulation contrast microscopy is that, by finely controlling the light that reaches the objective, it allows scientists to see individual slices of a specimen at a time, without being distracted by light originating from layers above or below. Such 'optical sectioning' can be performed again and again, until the scientist has viewed the entire depth of a specimen.

Unlike DIC microscopy, Hoffmann modulation contrast microscopy is not confused by birefringent specimens, since the crossed polarizers are not positioned either side of a specimen but – if at all – beneath it. Also unlike DIC microscopy, it can work with specimens housed in either glass or plastic dishes.

Fluorescence microscopy

Hoffman microscopy – like DIC microscopy, polarized-light microscopy and all the other types of microscopy described above – achieves quality images by carefully manipulating the light that illuminates and passes through a specimen. But there is a broad class of microscopy for which the images are not a direct product of the illuminating light at all. This class is called fluorescence microscopy, and it relies on the ability of certain substances not to reflect or diffract the illuminating light, but to be stimulated into emitting light themselves at a different wavelength. The primary light that you see in fluorescence microscopy is not the illuminating light, but the light generated by the specimen under study. To ensure the light is emitted at visible wavelengths, the illuminating light must be at shorter wavelengths.

Fluorescence microscopy usually requires specialist fluorescence microscopes, although the advantages of such microscopes are many. They are very sensitive and can provide very high-contrast images of bright specimens on a dark background. Most importantly, they can be very selective. Rather than observe every part of a specimen, a scientist can concentrate on specific features or components that he or she has labeled with a fluorescent probe, or 'fluorophore'.

Fluorescence microscopy has a long history, beginning in 1911 when the first successful fluorescence microscope was created by the physicist Oskar Heimstädt. In Heimstädt's microscope, the illuminating light was filtered so that only the short-wavelength ultraviolet light remained, while the direct portion was occluded, as in dark-field microscopy, to increase contrast. The sensitivity was such that Heimstädt managed to image bacteria, although the process was not especially straightforward. For mainstream fluorescence microscopy, scientists had to wait for the development

of ***epi-fluorescence microscopy*** in 1929 by the German pharmacologist Philipp Ellinger and the German anatomist August Hirt.

The epi-fluorescence microscope is sometimes also called an incident-light fluorescence microscope, because the excitation light source is on the same side of the specimen as the objective and the eyepiece. In this more efficient arrangement, the excitation light is directed through the objective to the specimen, which fluoresces visible light back again. The excitation light is excluded from the final image by a specific filter, a dichroic mirror, which reflects or transmits only a precise, narrow range of wavelengths.

The main restriction with fluorescence microscopy is that the intensity of the excitation light has to be as low as possible in order to prevent it damaging the specimen via phototoxicity, but this means that the resultant fluorescence signal is usually very weak. Hence the objectives for fluorescence microscopy need to possess the highest possible NA values, as well as the highest transmission values, throughout the spectrum of light.

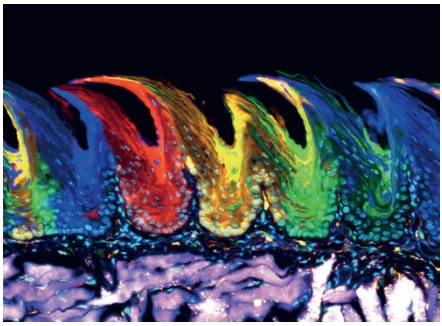

Figure 10. Rainbow mouse. Each interpapillary pit of the tongue is occupied by single-color cells that originate from monoclonal stem cells. Five colors: four fluorescent protein (EGFP, mCerulean, mOrange, mCherry) and nuclear staining of Hoechst, imaged by fluorescence microscopy. Adapted with permission from Macmillan Publishers Ltd: Tanaka T. Komai Y, Tokuyama Y, *et al. Nature Cell Biology* 2013;15:511–9, copyright 2013

Confocal microscopy

The resolution of epi-fluorescence microscopes is limited, however, because the images are fogged by light emitted by parts of the specimen in out-of-focus planes. Trying to remove this out-of-focus background light is what led the American artificial intelligence scientist Marvin Minsky to invent ***confocal microscopy*** in 1957, although its development into a usable fluorescence technique would take several more decades.

Today, there are various types of confocal microscopy, but the idea behind all of them is to illuminate just a single point of a specimen – with a laser, for example – and then limit the light transmitted through the objective using a pinhole. Since this process reduces the image to a single point too, it must be repeated again and again over the surface of a specimen (scanning), and the results captured with detectors such as a camera or a photomultiplier tube until an image of the entire specimen is revealed. For these reasons, confocal microscopes are expensive, stand-alone devices.

Confocal microscope objectives have similar requirements to the objectives used for fluorescence microscopy. As the resolution

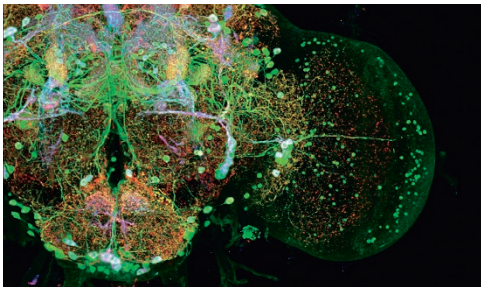

Figure 11. Nerve cells in a fruit fly brain imaged by confocal microscopy (three different color fluorophores have been used to label cell bodies and neurites). Courtesy of Jun Tanimura and Kei Ito, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan

in the vertical direction, known as the z axis, is much higher than in wide-field fluorescence microscopy, the chromatical aberration is of highest importance. Multi-color images taken with the wrong objective will show a spread of colors along the z axis due to the shift of focal point along the optical axis. This makes it impossible to perform three-dimensional rendering of the image or even quantitative analysis.

Total internal reflection fluorescence microscopy

Another way to remove the out-of-focus background light is to illuminate a specimen with a laser beam from the side. If the angle is sufficiently oblique to the optical axis, the light will enter the cover-slip and bounce back and forth between the upper and lower surface of the glass, a phenomenon known as total internal reflection. Crucially, however, not all the light is contained – a small amount of 'evanescent' light creeps beyond the cover-slip, into a specimen that has been prepared with fluorophores. Because this illuminating light is limited to a depth of no more than 200nm, there is no stray fluorescence to confuse the final image. This technique became known in the 1980s as *total internal reflection fluorescence (TIRF) microscopy*. Although it is often performed on

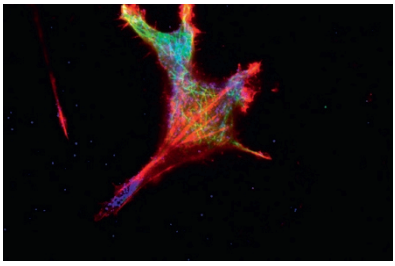

Figure 12. Actin imaged by TIRF microscopy using three different color fluorophores. Image courtesy of M. Faretta, D. Parazzoli, IEO-IFOM, Milano.

dedicated instruments, it can also be performed less expensively on modified epi-fluorescent microscopes.

Because the illuminating light has to be at a certain angle to the optical axis to produce total internal reflection, a TIRF objective has to have an NA of at least 1.42. Higher NAs will give the user the freedom to slightly change the incident light angle and thus modify the penetration depth of the evanescence wave. Typical TIRF objectives have an NA of at least 1.45 and can even reach up to 1.7, although this requires the use of a special immersion oil and cover glasses.

Light-sheet microscopy

A recently introduced technique that bears some similarity to confocal and TIRF microscopy is ***light-sheet microscopy*** (which is the subject of a separate Essential Knowledge Briefing). Although the basis of light-sheet microscopy was first laid out in the 1900s, it would not see widespread laboratory use until after the turn of the millennium. Like confocal and TIRF microscopy, light-sheet microscopy removes out-of-focus light from the final image, but it is also particularly good at optical sectioning.

Light-sheet microscopy works by focusing a laser beam in only one direction (as opposed to the usual two dimensions) and directing it to the specimen from the side, perpendicular to the axis of observation. This produces a thin sheet of light that excites fluorescence in just a slice of the specimen. Steadily moving this 'light sheet' through the specimen produces a stack of images that can be combined into a single three-dimensional image with a fair resolution. Light-sheet microscopy can be performed inexpensively by setting up individual components in a lab, or it can be performed on a dedicated instrument.

Multiphoton microscopy

There is one final specialist fluorescence technique that can deliver crisp images, free from out-of-focus blur, from deeper within specimens than is possible with other microscopy techniques. It relies on a quantum phenomenon first observed in the early 1960s, in which a fluorophore absorbs not just one photon of illuminating light, as in conventional fluorescence, but two simultaneously. The result is still the re-emission, or fluorescence, of one photon, but the energy of this photon is approximately the sum of the energies of the two illuminating photons – and its wavelength approximately half of them.

Because of this energy–wavelength relationship, ***two-photon excitation microscopy*** requires the illuminating light to have a wavelength roughly twice as long (and an energy roughly half as much) as usual, which means in the infrared part of the spectrum. This is a great advantage, however, because infrared light can penetrate further into tissue without scattering than ultraviolet light, allowing scientists to witness biological processes occurring deep within a specimen. Infrared light also poses less risk of damaging a specimen, which is particularly important when imaging living tissue.

Despite relying on a fairly well-established phenomenon, two-photon excitation microscopy took a while to be developed because it requires an intense illuminating light in order to raise the probability of two photons being absorbed simultaneously. Such light sources became available in the 1990s, with focused laser beams that could emit intense bursts of photons for less than one trillionth of a second at a time. More recently, researchers have developed laser sources that can provoke three-photon absorption for microscopy, extending the depth of specimen penetration even further.

With the multiphoton effect, the resultant fluorescence comes from a single focal plane, regardless of the color. Because of this, a multiphoton-dedicated objective does not need high chromatical aberration correction, but rather requires a high NA to collect as much fluorescence signal as possible. Typically, multiphoton microscopy is performed deep inside the tissue and so the fluorescence light is highly scattered by the specimen; this means the field of view has to be as high as possible to catch the maximum number of photons emanating from the specimen.

The objective also needs to collect as much excitation light as possible to ensure the highest resolution and efficiency. Thus the back focal NA has to match the diameter of the infrared laser beam to ensure that no light is lost on the excitation side.

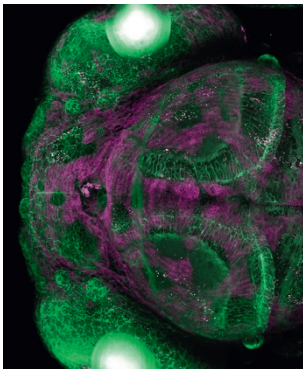

Figure 13. Transgenic zebrafish imaged by multiphoton microscopy. Image courtesy of Dr Rachel O Wong, Mr Philip Williams

CASE STUDY: Mapping mouse brains

To understand how brains work, scientists need to know how the individual nerve cells, or neurons, are wired together. This is not an easy task: a human brain has roughly 100 billion of them, and even a mouse – a typical mammalian laboratory subject – has upwards of 70 million. Worse, their arrangement and connections are not fixed, but change every minute. For that reason, some scientists are attempting instead to draw a 'statistical' map of a brain's neurons. Such a map is not a replica of a brain, but rather provides a general understanding of how different neurons are arranged by type. 'Our contribution to the scientific community is to provide a technique that can, in a reasonably short time, reveal the spatial arrangement of different classes of neurons throughout the brain,' explains biophysicist Ludovico Silvestri of the University of Florence in Italy.

To draw a statistical map, Silvestri needs to observe the neurons in every layer of a brain. Since you cannot literally slice up a brain without destroying it, Silvestri has turned to light-sheet microscopy, which allows him to quickly record clear two-dimensional pictures of a mouse brain, one layer at a time, without physically sectioning the tissue. He then uses a computer program to record the number of neurons and their arrangement.

This year, Silvestri used light-sheet microscopy to characterize the spatial distribution of Purkinje'cells – a class of neuron – in a mouse's cerebellum, demonstrating that the technique works in practice. He hopes that a complete statistical map of a mouse brain will help other scientists to create artificial neural networks that mimic its function.

Silvestri L, Paciscopi M, Soda P, et al. *Quantitative neuroanatomy of all Purkinje cells with light sheet microscopy and high-throughput image analysis.* Front Neuroanat *2015;9:68 (http://dx.doi.org/10.3389/fnana.2015.00068).*

FURTHER INFORMATION

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